Calcium Inhibits Renin Gene Expression by Transcriptional and Posttranscriptional Mechanisms

Jürgen Klar, Martin Sigl, Birgit Obermayer, Frank Schweda, Bernhard K. Krämer, Armin Kurtz

Abstract—The aim of this study was to investigate the role of cytosolic calcium for renin gene expression in juxtaglomerular cells. For this purpose, we used the immortalized juxtaglomerular mouse cell line As4.1. To increase cytosolic calcium concentration, we treated the cells with thapsigargin and cyclopiazonic acid, inhibitors of the endoplasmatic reticulum Ca2+ ATPase. Thapsigargin and cyclopiazonic acid inhibited renin gene expression in a characteristic time and concentration-dependent manner. This effect was concentration-dependently blocked by BAPTA-AM, an intracellular Ca2+ chelator. Pharmacological blocking of protein kinase C activity by calphostin, Gö6976, and Gö6983 did not change the effect of thapsigargin on renin gene expression. Experiments with renin1C–promoter–reporter constructs revealed that thapsigargin inhibited renin gene transcription. Analysis of deletion constructs of the renin1C promoter indicated that regulatory elements involved in the calcium-mediated inhibition of renin gene transcription are located in the enhancer region of the renin gene and that ≥3 transcription factor-binding sites are involved in this process. In addition, thapsigargin reduced the renin mRNA half-life from 10 hours (control conditions) to 4 hours. Knockdown studies with small interfering RNA directed to dynamin-1 mRNA revealed that dynamin-1 is likely to be involved in the calcium-mediated destabilization of renin mRNA. These data suggest that calcium inhibits renin gene expression in juxtaglomerular cells via a concerted action of inhibition of renin gene transcription and destabilization of renin mRNA. (Hypertension. 2005;46:1340-1346.)

Key Words: calcium ■ renin-angiotensin-aldosterone system ■ thapsigargin ■ mRNA stability

The renin-angiotensin-aldosterone system is a major regulatory system controlling extracellular fluid volume and blood pressure. The activity of the renin-angiotensin-aldosterone system is rate limited by the activity of the protease renin. Renin itself is also regulated by a variety of factors. In the past few years, our knowledge has increased substantially about the regulation of renin gene expression at the cellular level. For the transcription of the mouse renin gene, 2 critical regions, a proximal promoter region at −197 to −50 bp and an enhancer element at −2866 to −2625 bp, have been identified. Also, the regulation of the renin mRNA stability is influenced by various factors, among these, interacting partner proteins like MINT, dynamin, or nucleolin have been found to be involved in the stabilization process of the renin mRNA.

The focus of our study was to investigate the role of calcium in the regulation of renin gene expression. This is of particular interest, because vasoactive peptides like angiotensin II or endothelin-1, which are known to be direct regulators of renin gene expression, activate the phospholipase C via a G protein-coupled receptor and, in turn, increase inositol triphosphate and diacylglycerol (DAG). Whereas DAG is known to activate protein kinase C, inositol triphosphate increases the cytosolic calcium concentration [Ca2+]i. However, it is still a matter of controversy regarding which one of these intracellular factors translates into an inhibitory signal on renin gene expression in juxtaglomerular cells. Ryan et al have reported that endothelin-1 increases the intracellular calcium concentration in As4.1 cells and that this increase is paralleled by a decrease in the renin gene transcription. Pan et al provided evidence that a HOX.PBX binding site at −60 bp and also the enhancer region of the renin gene are involved in the negative regulation of the renin gene by endothelin-1. Studies with the human renin promoter showed that increases in the cytosolic calcium concentration are accompanied by a binding of the ref-1 transcription factor to a nCaRE sequence in the renin promoter in choriodecidual cells.

Therefore, we investigated the effect of calcium on renin gene expression and addressed the intracellular mechanisms involved in this process. For this purpose, we used the immortalized juxtaglomerular cell line As4.1. To increase cytosolic calcium concentration, we used inhibitors of the endoplasmatic reticulum calcium ATPase. From our data, we infer that an increase in the intracellular calcium concentration decreases renin gene expression. This process is independent of protein kinase C activity. This downregulation of renin...
gene expression is mediated by inhibition of renin gene transcription and by a destabilization of the renin mRNA.

Methods

As4.1 Cell Culture
The renin-expressing As4.1 cell line was obtained from the American Type Culture Collection (ATCC No. CRL2193). Cells were cultured as described.9,10

RNA Interference
Ready-to-use double-stranded 21-nucleotide small interfering RNAs (siRNAs) were synthesized by IBA. The siRNA targeting ref-1 (GenBank accession no. NM_009687) corresponded to position 94 to 113 relative to the first nucleotide of the start codon. The siRNA targeting dynamin 1 (GenBank accession no. L31397) corresponded to position 41 to 60 relative to the first nucleotide of the start codon. Single-stranded sense RNA (used as control) or siRNA (200 nM) was transfected in As4.1 cells with oligofectamine (Invitrogen) according to the manufacturer’s protocol. Cells were harvested 72 hours after transfection.

RNA Isolation and Ribonuclease Protection Assay
Total RNA was isolated from As4.1 cells using Qiagen RNeasy Spin Columns. RNA protection assay was done as described.11

Reverse Transcriptase Reaction and Quantitative PCR Analysis
Reverse transcription and quantitative PCR analysis was performed as described.10 The primers for renin and β-actin were used as described.11 The sequences of the primers used for the amplification of dynamin1, dynamin2, MINT, and nucleolin are available on request.

Plasmid Constructs
The 4.2-kb, 2.8-kb, and the deletion constructs of the 2.8-kb renin promoter fragment were obtained as described previously.11 The 3 TGAC sites from −2698 to −2695 bp, from −2674 to −2671 bp, and from −2657 to −2654 bp in the 2.8-kb fragment were deleted with the QuikChange site-directed mutagenesis kit (Stratagene).

Transient DNA Transfection
Transfection was performed using Fugene 6 transfection reagent (Roche Applied Science) essentially as described.11

Dual Luciferase Assay
Luciferase activity was measured with the Dual Luciferase Assay kit from Promega according to the manufacturer’s instructions. Light production was measured for 20 s for firefly luciferase and renilla luciferase activity in a Lumat LB 9507 luminometer (Berthold). The relative luciferase activity was calculated as luciferase/renilla luciferase.

Electrophoretic Mobility-Shift Assay
Nuclear protein extracts were obtained as described.11 The gel shift reaction (10 μL) was run as described.11 After electrophoresis at room temperature, the gels were dried for autoradiography.

Used Oligonucleotides
The following oligonucleotides were used: probe A, 5’ ATCCTC-CCAAAATGACATCACTAACCCAG 3’; mutated probe A, 5’ ATCCCTCCAAAATGACATCACTAACCCAG 3’; probe B, 5’ ACGCAG-ATGTTGACCTGGCTGACTCTT 3’; mutated probe B, 5’ ACGCAGATGTAATTTCTGCTGACTCTT 3’; probe C, 5’ GCCGTACCTGACCTCTGAGTGCGCTG 3’; and mutated probe C, 5’ GGGCTGACTCAATTTGACTGGCGCTG 3’.

Immunoblotting
Total protein extraction from As4.1 cells and Western blotting was performed as described.11 Antidynamin 1 antibody (ab14448) was purchased from Abcam.

Statistics
Data are expressed as mean±SE. To test homogeneity of variance, data were analyzed by Levene’s test. Multiple comparisons of several groups were done by ANOVA followed by a Bonferroni reduction. *P<0.05 was considered significant.

Results
Effects of Ca2+ ATPase Inhibition on Renin Expression of the Mouse Juxtaglomerular Cell Line As4.1
To determine the effect of thapsigargin on renin gene expression, As4.1 cells were incubated with 100 nM thapsigargin for different durations with a maximum of 24 hours. Figure 1 shows that basal renin mRNA levels decrease in the presence of thapsigargin with a half-life of ~4 hours, reaching levels that were ~25-fold lower than control levels after 24 hours. The inhibitory effect of Ca2+ ATPase blockade by thapsigargin on renin mRNA was concentration dependent with a half-maximal effect at concentrations between 10 and 100 nM (data not shown). A downregulation of renin gene expression was also observed after blocking the Ca2+ ATPase with 10 μmol/L cyclopiazonic acid (Figure 2A). The decrease of renin mRNA levels in As4.1 cells after incubation with 100 nM thapsigargin or 10 μmol/L cyclopiazonic acid was blocked by BAPTA-AM (10 and 30 μmol/L), an intracellular Ca2+ chelator in a concentration-dependent fashion (Figure 2A).

Characterization of the Effect of Increased Intracellular Ca2+ Concentration on Renin Gene Expression
To exclude that the increase in the cytosolic calcium concentration evoked by thapsigargin may activate protein kinase C,
and protein kinase C activity, in turn, was responsible for the
downregulation of the renin mRNA abundance, we blocked
protein kinase C activity. For this purpose, we used 1 μmol/L
Gö6976, 1 μmol/L Gö6983, and 100 nmol/L calphostin, which
are all well-established potent inhibitors of protein kinase C
activity. As4.1 cells, therefore, were incubated with the
inhibitors alone and in combination with thapsigargin for 24
hours. The determination of renin mRNA abundance revealed
that the downregulation of the renin mRNA expression by
thapsigargin is not altered by any of the used inhibitors
(Figure 2B). From this we conclude that thapsigargin could
act on renin gene expression independent of protein kinase C
activity.

Mechanism of Action of Increased Intracellular
Ca\(^{2+}\) Concentration on Renin Gene Transcription
To additionally study downstream signaling pathways by
which Ca\(^{2+}\) exerts its action on renin gene expression, we
tested whether this effect may result from a decreased renin
gene transcription. We, therefore, transfected As4.1 cells with
mouse renin\(^1\) promoter luciferase gene constructs. It turned
out that 100 nmol/L of thapsigargin reduced the activity of a
4.2-kb renin promoter fragment, as well as a 2.8-kb renin
promoter fragment to ~30% to 40% of the control value (data
not shown). To narrow down the possible sequences mediating
this effect of thapsigargin on renin gene promoter activity,
different constructs of the 2.8-kb renin promoter fragment in
which different deletions were set were tested for transcriptional
activity. All of the constructs displayed similar basal
transcriptional activity except that of construct 2.8kb
\(\Delta\)2651 to \(\Delta\)2603 where a nearly complete lack of transcriptional
activity was found. The effect of thapsigargin on renin
promoter activity was blunted when the region from \(\Delta\)2719
to \(\Delta\)2651 and from \(\Delta\)2651 to \(\Delta\)2603 was deleted, indicating
important regulatory sequences for Ca\(^{2+}\)-mediated inhibition
of renin gene transcription in the area from \(\Delta\)2719 to \(\Delta\)2603
(Figure 3).

This area is part of the enhancer region of the renin
gene,\(^4\), and a great many potential transcription binding
sites were described for this short DNA fragment. The
CAMP responsive element (CRE) site from \(\Delta\)2698 to
\(\Delta\)2691 (A) was found to play a crucial role in the
downregulation of renin gene expression by tumor necrosis
factor \(\alpha.\)\(^{15}\) Therefore, we investigated whether this CRE
site is also important for the downregulation of renin gene
expression by thapsigargin. As shown in Figure 4, the
deletion of the CRE site from \(\Delta\)2698 to \(\Delta\)2694 (A) alone
had only a marginal effect on the inhibition of renin gene
transcription.
transcription by thapsigargin. As soon as 2 additional TAGC sites from −2674 to −2671 (B) and from −2657 to −2654 (C) were deleted, the regulatory effect of thapsigargin on renin gene transcription was missing, although the deletion per se had no inhibitory effect on renin gene transcription (Figure 4). Gel shift assays with nuclear extracts from As4.1 cells treated without (control) or with 100 nmol/L thapsigargin showed that every single of the 3 TGAC sites can bind nuclear proteins, but it also revealed that no changes in the DNA-protein complex, which is formed with each of the 3 TGAC sites, is independent of whether nuclear extracts were derived from control or thapsigargin-treated cells (Figure 5).

For the human renin promoter, it was speculated that ref-1, a calcium-regulated protein, is involved in the regulation of the renin gene transcription by endothelins. We, therefore, tested whether ref-1 is also involved in the downregulation of renin gene expression mediated by calcium. We also found that the ref-1 protein is expressed in the nucleus of As4.1 cells. Knockdown experiments with siRNA for ref-1 mRNA reduced ref-1 mRNA expression to 10% of cells transfected with the sense siRNA as a negative control but had no influence on the downregulation of renin mRNA by thapsigargin (data not shown).

**Mechanism of Action of Increased Intracellular Ca²⁺ Concentration on Renin mRNA Stability**

By blocking transcription in As4.1 cells with 2 μmol/L of actinomycin D or 20 μmol/L of α-amanitin, we determined that the renin mRNA half-life time was ≈10 hours (Figure 6). In contrast, in As4.1 cells, which were treated with 100 nmol/L of thapsigargin, we calculated a renin mRNA half-life time of ≈4 hours (Figure 6). Renin mRNA degradation in thapsigargin-treated cells is, therefore, ≈2.5 times faster than in untreated cells. From this finding, we concluded that thapsigargin also influences renin mRNA stability.

The involvement of many proteins, like dynamin nucleolin or MINT-homologous proteins, in the stabilization process of the human renin mRNA has been reported by several different groups. We investigated whether 1 of these proteins might be involved in the destabilization process of the murine renin mRNA mediated by thapsigargin. We found that the mRNA expression of dynamin-1 was time-dependently reduced by thapsigargin (Figure 7), whereas the mRNA expression of other proteins, possibly involved in the stabilization of renin mRNA, was not altered (data not shown). Additional studies revealed that a specific knockdown of the dynamin mRNA by siRNA also reduced dynamin-1 protein expression in As4.1 cells (Figure 8A). Because a specific knockdown of the dynamin-1 mRNA results in a reduced renin mRNA expression in As4.1 cells, it might be
assumed that dynamin-1 is directly involved in the down-regulation of renin gene expression via a destabilization of the renin mRNA (Figure 8B).

**Discussion**

Our study shows that thapsigargin, which inhibits the endoplasmatic Ca\(^{2+}\)/H\(_{\text{ATPase}}\), and, therefore, increases the cytosolic calcium concentration, downregulates renin gene expression in a juxtaglomerular cell line. From this, we suggest that an increase of cytosolic calcium concentration can inhibit renin gene expression. This conclusion is supported by the fact that inhibition of the renin gene expression is also found by inhibiting the endoplasmatic Ca\(^{2+}\)/H\(_{\text{ATPase}}\) with cyclopiazonic acid and by the fact that the effect of both pharmacological agents on renin gene expression can be blocked with the intracellular Ca\(^{2+}\) chelator BAPTA-AM. Furthermore, our findings indicate that the inhibitory effect of increased calcium concentrations on renin gene expression is independent of the protein kinase C activity. This finding fits well with previous results showing that inhibition of renin gene expression by endothelin-1 is accompanied by an increased cytosolic calcium concentration in As4.1 cells. Beyond this, our data suggest that an increased calcium concentration inhibits renin gene expression by an inhibition of renin gene transcription and by accelerating the decay of renin mRNA. Concerning renin gene transcription, the effect of thapsigargin is mediated by transcription factor-binding sites located in the enhancer region of the renin gene. Other than the core promoter site from −117 to −1, the enhancer of the renin gene constitutes the most important regulatory element for mouse renin gene transcription. Because of the fact that the deletion of the enhancer structure completely blunted the effect of thapsigargin on renin gene transcription, we focused our additional studies on the enhancer region. It turned out that the effect of thapsigargin on renin gene transcription is not mediated via a single transcription factor-binding site. In contrast, our findings suggest that auxiliary transcriptional proteins be involved in the inhibitory effect of thapsigargin. This is comparable to findings of the inhibitory effect of
cytokines like interleukin 1β, interleukin 6, or oncostatin M, which also exert their inhibitory effects on renin gene transcription by ≥3 transcription factor-binding sites. These responsible elements in the renin enhancer have already been described to play a crucial role for renin gene transcription. The site from −2698 to −2694 bp was found to bind the CREB/CREM transcription factor and to respond to forskolin treatment in juxtaglomerular cells.11,18 It was shown that the inhibition of renin gene transcription by tumor necrosis factor α is indirectly mediated via this CRE site.15 The sites from −2674 to −2671 bp and from −2657 to −2654 bp are described as TGACCT motifs, which are homologous to the steroid receptor-binding site. Retinoic acid receptors/retninoic X receptors have been identified as transcription factors binding to these 2 sites.19 In addition, also the nuclear orphan receptor EAR2 has been shown to bind to the TGACCT motif.20 Although retinoic acid is responsible for upregulation of renin gene expression, the EAR2 regulates the renin gene expression in As4.1 cells in a negative way.19,20 Li et al21 demonstrated that renin mRNA downregulation by vitamin D in As4.1 cells results from a decrease in the ren1 receptor activity. In addition, Shi et al19 demonstrated that the transcriptional activity of a construct containing a triple copy of a TGACCT-N10-TGACCT motif placed upstream of the renin promoter is repressed by 1.25 dihydroxyvitamin D3. A sequence highly homologous to the mouse renin enhancer has been found in the human renin promoter, which is located ∼11 kb upstream of the transcriptional start site and shares 71% identity with the mouse renin enhancer. From these findings, we suggest that our conclusions for the transcriptional regulation of the mouse renin transcription may also be relevant for the regulation of the human renin transcription. However, it has been suggested that the regulation of the human renin gene transcription by endothelins in choriodecidual cells is regulated via increasing the intracellular calcium concentration and thereby activating the ref-1 protein, which, in turn, inhibits renin gene transcription.8 We also found expression of the ref-1 protein in the mouse juxtaglomerular cells, but a knockdown of the ref-1 mRNA and the ref-1 protein had no effect on renin gene expression. In view of these data, we suggest that it is not likely that ref-1 is crucially involved in the calcium-mediated downregulation of the renin gene transcription in As4.1 cells.

In addition to the regulation of the renin gene expression at the transcriptional level, we found that thapsigargin also downregulates renin gene expression by destabilization of the mRNA. The first evidence in support of an additional effect of thapsigargin on renin gene expression other than modulating renin gene transcription was the findings of a shortened half-life of renin mRNA in thapsigargin treated As4.1 cells (t1/2=4 hours) compared with controls (t1/2=10 hours). The involvement of a stabilization process in the regulation of the renin mRNA levels was already described some years ago for the cAMP-induced up regulation of the renin gene expression.22 In addition, some proteins have recently been identified binding to the renin mRNA and thereby playing an important role in the stabilization of the renin mRNA.16 For example, knockdown experiments for the protein HuR revealed that the half-life of human renin mRNA is decreased from 4.5 to 2.5 hours.23 However, in addition to stabilization proteins, proteins that destabilize human renin mRNA were also found. So, the knockdown of HABHB revealed an increased half-life of the human renin mRNA to ∼12 hours. In addition, for the human renin mRNA, a number of RNA-protein complexes were identified that increased when the cells were treated with forskolin, which, in turn, increases the intracellular cAMP concentration.26 In the mouse juxtaglomerular cell line, we found the expression of mRNAs for dynamin-1, for dynamin-2, and for MINT but not for nucleolin, all proteins that are considered members of the human renin mRNA-protein complex.25 Our findings also reveal that, among these proteins, dynamin-1 is selectively downregulated by thapsigargin. This finding could point toward a role for dynamin-1 in the destabilization process of the renin mRNA mediated by thapsigargin, and this assumption is confirmed by our finding that the knockdown of dynamin-1 mRNA by siRNA also results in an inhibition of renin gene expression.

**Perspectives**

Although it has been known for a long time that angiotensin II and endothelins inhibit renin gene expression in juxtaglomerular cells and that this decrease is accompanied by an increased protein kinase C activity and by an increase in intracellular calcium concentration, the additional intracellular pathways are still a matter of controversy. Our data show that the inhibition of renin gene expression by an
increased cytosolic calcium concentration is a complex molecular process and results in a combined effect of destabilization of the renin mRNA and inhibition of renin gene transcription via transcription factor binding sites located in the renin enhancer. In additional studies, the proteins that are involved in this transcriptional repression of renin gene expression and how these proteins are regulated and altered by the cytosolic calcium concentration have to be discovered.

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